CONFIDENTIAL

DATA EVALUATION REPORT

Reviewed by: Michael Watson, Ph.D., Plant Pathologist MTW MAR 29 1999

Secondary Reviewer: John Kough, Ph.D., Biologist JJK

STUDY TYPE:	Analysis of Samples (151A-13) Certification of Ingredient Limits (151A-15) [amendment No. 2 to MRID no. 437634-02]
MRID NO:	446261-01
TEST MATERIAL:	*Aspergillus flavus* isolate AF 36
PROJECT NO:	IR-4 PR No. 52B
SPONSOR:	William L. Biehn, IR-4, North Brunswick, NJ & USDA-Southern Regional Research Center
TESTING FACILITY:	USDA-Southern Regional Research Center
TITLE OF REPORT:	*Aspergillus flavus* isolate AF36 - Analysis of Samples, Certification of Ingredient Limits, Analytical Methods for Certified Limits. Amendment No. 2 to MRID No. 43763402.
AUTHOR(S):	Dr. Peter J Cotty & Mr. Larry Antilla
STUDY COMPLETED:	June 25, 1998
CONCLUSION:	The description of the quality control and certification of limits procedures contained in this report are mostly acceptable. However, there are some steps in the described protocol which need further clarification or elaboration. These include: 1) Aflatoxin Production: the protocol describes use of TLC for aflatoxin detection. However, the description only mentions "aflatoxin standards" to be placed on the TLC plates and does not describe exactly what standards will be used. These standards and the level of detection should be identified; 2) Identification of *A. flavus* in Final Product: six of the thirty-six seeds are removed to vials containing water, and the conidia removed are used for vegetative compatibility analyses and spore producing ability in the final product: a) how much water is in each vial and does the amount of water affect the detection of non-vegetatively compatible *A. flavus* (i.e., detection limit)?; b) for mutant generation, after sectors have formed on the Rose Bengal Selection Medium, what "second" selection media is used for these sectors?; c) is variability in spore viability produced by the final product a consideration?
CLASSIFICATION:	SUPPLEMENTARY. Can be upgraded to ACCEPTABLE with adequate description/clarification of the deficiencies described above.
GOOD LABORATORY PRACTICE:	This study was not conducted in compliance with Good Laboratory Practice guidelines.

NOTE: This is amendment #2 to MRID# 437634-02. A subsequent amendment (#3 - MRID# 447137-01) has also been submitted and describes the new manufacturing process, for which these quality control processes are included.

I. STUDY DESIGN

Test Material: Sterilized wheat seeds colonized by *Aspergillus flavus* isolate AF36. The end-use product contains approximately 28 sterile wheat seeds, colonized by *A. flavus* AF36 per gram. Each gram of seeds is capable of producing at least 2.5 billion spores when inoculated at 31° C, 100% RH for 7 days.

Methods: *Identification of Aspergillus flavus AF 36 prior to wheat inoculation.* The *A. flavus* AF36 is transferred from either silica gel or culture vials to petri dishes containing 5/2 agar, Nutrient Agar (NA) and Czapeks-Dox agar (CD). The NA and CD plates are visually examined to ensure that the cultures are uniform and free of bacterial and fungal contaminants. After incubation for 5 to 7 days at 31° C, the working cultures are made by removing plugs from each 5/2 plate and aseptically transferring them to 10, 20 ml vials containing 5 ml distilled water each. Aflatoxin producing potential of each suspension is determined (as described below) and a vegetative compatibility study is performed to confirm the AF36 strain of *A. flavus*. The working culture vials are used to inoculate the 5/2 plates from which the conidial suspension used to inoculate the sterile wheat seed is produced.

Aflatoxin Production: Aflatoxin production is assessed in a medium containing the following (per liter): 50.0 g Sucrose, 3.0 g NH_4 So_4, 10 g KH_2 PO_4, 2.0 g $MgSO_4$-$7H_2$ O, 0.7 mg Na_2 B_4 O_7-10 H_2O, 0.5 g mg $(NH_4)_6$ Mo_7 O_{24}-$4H_2O$, 10.0 mg $Fe_2(SO_4)_3$-$6H_2O$, 0.3 mg $CuSO_4$-$5H_2$ O, 0.11 mg $MnSO_4$-H_2 O, 17.5 mg $ZnSO_4$-$7H_2$ O. The medium is adjusted with either NaOH or HCl to pH 4.75 prior to autoclaving. Erylenmyer flasks (250 ml) containing 70 ml of medium are inoculated with approximately 5 x 10^3 spores per milliliter of the working culture to be tested and incubated on a shaker at 150 RPM, 31° C, in the dark for 5 days. After incubation, 70 ml of acetone is added to each flask to lyse fungal cells and extract aflatoxins from the mycelium. The flasks are allowed to sit at room temperature for 1 to 4 hr and then are briefly swirled to mix the contents. Four microliters of each culture is spotted on Thin-Layer Chromatography (TLC) Plates (silica gel 60, 250 mm) with aflatoxin standards and separated by development with diethyl ether-methanol-water (96:3:1). The plates are observed under long-wave (365 nm) uv light in a chromatography view box for the presence of aflatoxins

If the culture is negative for aflatoxins, it is filtered through number 4 Whatman paper. Following filtration, 100 ml of the filtrate is added to an equal volume water in a 250 ml separatory funnel and the solution is extracted twice with 25 ml methylene chloride. The methylene chloride extracts are filtered through 50 g

anhydrous sodium sulfate to remove residual water and the sodium sulfate extracts are combined, evaporated at room temperature and the residual is dissolved in 4 ml methylene chloride and again tested by TLC for the presence of aflatoxins as described above. If the cultures are again negative for aflatoxins, the extract is evaporated to dryness and reconstituted in 500 µl and again tested as described above for the presence of aflatoxins. If the TLC plates are again negative for aflatoxins, it is determined that the cultures did not produce aflatoxins.

Final Product Quality Assurance:

Each batch of final product is analyzed for quantity of *A. flavus* conidia it will produce, and for microbial contaminants. A batch is defined as the quantity produced from one conidial suspension (up to 5, 000 lb.).

Conidial Suspension: After drying, 36 seeds from each batch of product are placed in multiwell plates at 31° C & 100% relative humidity for seven days. After incubation, the seeds are examined for microbial growth and for seed colonization typical of *A. flavus*. Quantitative assessment of spore yield is by turbidity. Spores are washed from each seed with 95% ethanol (3X), and the washings are pooled. The ethanol volume from each of the pooled washings is increased to 11.5 ml, and the washings from two seeds is combined (23 total ml). Water is added to the pooled washings to bring the volume to 46 ml and turbidity is measured with a turbitometer. The turbidity measurement is compared to a standard curve [NTU (National Turbitometric Unit) vs spore concentration] in order to determine the spore yield. Six measurements per batch are performed and the average spore yield must exceed 2.5 billion spores per gram.

Identification of A. flavus in Final Product: Each of the 36 seeds used above are examined by eye after the incubation described above, to ensure homogenous colonization by *A. flavus*. The examination includes confirming the conidia present are typical of *A. flavus*, and to ensure that no other organisms are present. Six of the 36 seeds are individually, aseptically removed from the multiwell plates and placed in separate vials containing sterile water. The vials are agitated to release conidia and an aliquot (20 µl) of the conidial suspension from each vial is transferred to a single well (3 mm diameter) cut in the center of Rose Bengal Selection Medium in a 9 cm petri dish. The cultures are incubated for 5 to 14 days (without illumination) at 31° C. The petri dishes are periodically examined for formation of sectors, which are putative nitrate non-utilizing mutants.

The sectors which have formed are transferred to a second selection medium and incubated for 3 to 5 days to allow the mutant to outgrow the wildtype. Hyphal tips of the sectors are then confirmed to be nitrate non-utilizing on the basal medium containing nitrate as the sole nitrogen source. Mutants are then paired with tester mutants of *A. flavus* AF36. The two testers defining AF36 are stored long term on silica gel as VCG-A, T1 (*cnx*-) and VCG-A, T2 (*niaD*-). These cultures are

deposited at the ATCC (Rockville, MD) as ATCC 96045 and ATCC 96047 respectively. If the mutant complements one of the tester mutants, the conidia are *A. flavus* AF36 (see attached figure).

In summary, in this test, a mutant of an isolate is paired with tester mutants. If the isolate mutant receives genetic material from a tester to repair its mutation, a zone of thick growth occurs. This means that the isolate from which the mutant originated is in the vegetative compatibility group of *A. flavus* AF36.

Quality Control Procedures for Microbial Contaminants in the End-Use Product: Fifty seeds from each batch are placed aseptically on both Nutrient Agar and Violet Red Bile Agar (medium for coliforms). Plates are examined after five days for the presence of contaminants.

End-Use Product Specifications:
Coliforms (VRB): <2 of 50 seeds contaminated
Bacteria (Nutrient Agar): <6 of 50 seeds contaminated
A. flavus AF36 spore count: >2.5 x 10^9 spores per gram [capable of producing >2.5 x 10^9 *A. flavus* AF36 when incubated at 31° C and 100% relative humidity].

-> If a lot (batch) fails the above criteria in the initial quality control assays, the lot is reassayed for contaminants using twice as many seeds (i.e. 100 seeds instead of 50). If any of the contamination criteria are not met on reassay, the batch is discarded.
-> If the average spored yield of *A. flavus* AF36 does not exceed 2.5 x 10^9 spores/gm in the batch based upon the average of six measurements, the average spore yield is determined again using 12 measurements per batch. If the average spore yield is again not >2.5 x 10^9 spores/gm, the batch is discarded.

DISCUSSION

The description of the quality control and certification of limits procedures contained in this report are mostly acceptable. However, there are some steps in the described protocol which need further clarification or elaboration. These include:

1. Aflatoxin Production: a) the protocol describes the method to be used for detection of aflatoxin production. However, the protocol only mentions "aflatoxin standards" to be placed on the TLC plates and does not describe exactly what standards will be used. These standards and their source (i.e. supplier) should be identified; b) the level of detection of aflatoxins is not described. This information should be provided based upon tests using acceptable controls.
2. Identification of *A. flavus* in Final Product: in the sentence, "Six of the 36 seeds are individually, aseptically removed from the multiwell plates and placed in separate vials containing sterile water": a) how much water does

each vial contain? b) does the amount of water contained in each vial, and hence the dilution plated, affect the identification of vegetative compatibility/non-compatibility? i.e., what is the detection limit for strains of *A. flavus* other than AF36?; c) in the sentence, "The sectors which have formed are transferred to a second selection medium and incubated for 3 to 5 days to allow the mutant to outgrow the wildtype". What is the medium used here?; d) there is no indication in the protocol that spore viability is determined. Does the minimum spore yield of 2.5 billion spores/gram take into account variability in spore viability?

CLASSIFICATION - SUPPLEMENTARY. Can be upgraded to ACCEPTABLE with adequate clarification/description of the deficiencies described above.

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R141808

Chemical	Aspergillus flavus 36 colonized wheat seed
	PC Code: 006456
HED File Code:	41500 BPPD Tox/Chem
Memo Date:	3/29/1999
File ID:	00000000
Accession #:	000-00-9002

HED Records Reference Center
4/13/2007